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DEVELOPMENT OF MICROSPORE CULTURE TECHNOLOGY FOR THE PRODUCTION OF DOUBLED-HAPLOIDS IN CHICKPEAS

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Development of Microspore Culture Technology for the Production of Doubled-Haploids in Chickpea

Final Report on
ADF Project
No. 20010079

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A. ABSTRACT

The objective of this project was to develop doubled-haploid technology for chickpea (*Cicer arietinum* L.) using isolated microspore culture in collaboration with researchers from CLIMA (Australia). To date, a protocol was developed in which chickpea microspores could be switched from the gametophytic to the sporophytic pathway by applying a heat pre-treatment at the mono-nucleate stage. Induced microspores could then be regenerated to the cotyledonary embryo stage. However, conversion of embryos into plants could not be accomplished. Efforts continued to improve the androgenetic protocol and to identify critical parameters.

B. Executive Summary

This project involved the application of isolated microspore culture to chickpea (*Cicer arietinum* L.) in order to develop doubled-haploid technology. The project was conducted in close collaboration with Dr. J. Croser from CLIMA (Australia).

A multiple step protocol was developed in which chickpea microspores could be switched from the gametophytic to the sporophytic pathway by applying a heat pre-treatment at the mono-nucleate stage. Symmetrical microspore nuclei division was the first indicator of this switch. Subsequent culture of induced microspores in a liquid medium allowed the continued division of the microspore nuclei as well as the microspores themselves. As a result, pro-embryogenic clusters were obtained and transferred to various embryo development media on which embryos could be regenerated to the cotyledonary stage. However, plants could not be regenerated.

Extensive experiments were conducted in order to improve the androgenetic protocol and to identify critical parameters such as donor plant growth conditions, sterilization techniques, induction and development media, the gaseous atmosphere in the culture vessels, or the separation of the different microspore stages.
In addition, every step of the protocol was scrutinized in order to improve the conversion of embryos into plants. Centrifuging the pro-embryogenic clusters prior to transfer to embryo development media might have been the causal agent for the continued callusing by disturbing embryo polarity. However, these experiments could not be completed prior to the termination of this project.

The Australian Grain Research and Development Corporation (GRDC) are currently considering a continuation of the CLIMA project for another two years. In addition, four more years of funding has also been requested for their project from the Australian Research Council.

C. Abbreviations

CLIMA	Centre for Legumes in Mediterranean Agriculture, University of Western Australia, Crawley, Australia
ABA	Abscisic acid
BAP	6-Benzylaminopurine
4-CPA	4-Chlorophenoxy acetic acid
2,4-D	2,4-Dichlorophenoxy acetic acid
DAPI	4,6-Diamidino-2-phenylindole
GA_3	Gibberellic acid
2-HNA	2-Hydroxy nicotinic acid
IAA	3-Indole acetic acid
IBA	Indole butrytic acid
MES	2-(Morpholine) ethane sulfonic acid
NAA	Naphtalene acetic acid
PAA	Phenyl acetic acid
PGR	Plant Growth Regulators

D. Glossary

Stamen	Male part of a flower consisting of anther and filament
Anther	Male part of flower containing pollen
Pistil	Female part of the flower consisting of stigma, style and ovary with ovules
Microspore	Immature pollen with one nucleus

E. Technical Report

1. Project Background

Chickpea flowers contain female (pistil) and male (stamen) reproductive organs (Fig. 1). A part of the stamen called **anthers** produces pollen grains. As long as these pollen grains are immature, they are called **microspores** (see Diagram 2 in chapter 5). Pollen grains or microspores contain germ cells. Germ cells are unique in the life cycle of a plant because they contain only one (haploid) set of chromosomes, whereas all other plant cells are diploid i.e. they contain two sets of chromosomes, one originating from the male and one from the female germ cell. Given the appropriate conditions, plants can be regenerated directly from these germ cells and are consequently haploid as well. Upon doubling of the chromosome set, either spontaneously or by induction, a normal 'diploid' plant is regenerated except that the two chromosome sets are completely identical (homozygous). Hence, the plants derived from this pathway are called 'doubled-haploid' plants.

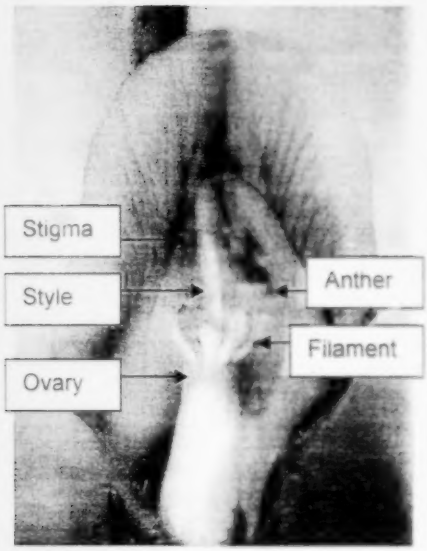


Fig. 1. Chickpea flower with male and female reproductive parts

The advantages of this technology are:

- Reduction in the time required for the development of new cultivars by 3-4 years. This would result in considerable savings to the breeder and the producer, particularly if an economy of scale can eventually be used to create an efficient system. High yielding, resistant cultivars would be available more quickly. This is especially important for keeping ahead of genetic changes in the *Ascochyta* blight fungus.
- Easier and faster identification of recessive genes and thus potentially more efficient selection for specific traits.
- Molecular-marker assisted selection becomes more efficient when used in conjunction with doubled-haploid plants.
- For genetic, mutagenesis, and transformation studies

This technology is now commonly used in crops such as canola, barley, and wheat. Doubled-haploid technology has not yet been developed for any cool season food legume and generally, pulse crops are considered recalcitrant in cell and tissue culture.

2. Project Objectives

The objective of this project was to develop doubled-haploid technology for chickpea (*Cicer arietinum* L.), specifically:

- To identify critical parameters in the development of a microspore protocol
- To regenerate microspore-derived embryos into plants

- To streamline this protocol to make it more cost-efficient
- To apply this technology to CDC breeding lines and to produce doubled-haploid seeds

3. Project Results and Discussion

A detailed description of our current microspore culture protocol is attached in a separate file (see Protocol Part I and II). It was developed for chickpea cultivars and breeding lines and was shown to be effective for induction. However, considerable variation existed between experiments as well as the very low embryo yield continued to be a major concern. Hence, improving the reproducibility and reliability of our protocol was one focus of our experiments; the second focus was on improving embryo development and conversion. Since numerous factors influence androgenesis and subsequent embryo development, each step of the protocol was scrutinized in order to determine key elements that affect the development of an efficient and reliable method for routine breeding purposes. Furthermore, the high cost associated with the development of doubled-haploid plants is a major factor for the limited application of this technology. Hence, finding ways to reduce costs was another objective of this project.

3.1. Donor Plants

Donor plant growing conditions have a profound influence on androgenesis. Any stress during this time will result in less success or complete failure of microspore induction and further regeneration (Wang *et al* 2000). Optimum conditions vary with genotype but are also affected by genotype x environment interactions (Powell 1990). Kasha *et al* (2001) strongly recommended avoiding any water or drought stress as well as pest infestations or spraying with pesticides. Hence, determining and then providing optimal growing conditions is a prerequisite for obtaining maximum microspore induction.

Soil Mixtures

Previous experiments had shown that a mixture of Redi-earth® and Sunshine® Mix No. 3 was an excellent substrate for growing chickpea. However, during the last year, we observed that some plants within a pot suddenly dried up and died within a few days. Close examination of the plants showed neither pest nor disease damage or root problems. Other groups working in the Phytotron had also reported similar problems with their plants. The Phytotron staff finally traced the problem back to the Redi-earth® mixture. The company admitted that they had added a highly phytotoxic detergent instead of a wetting agent to their product. Since the company was not willing to either refund or replace the soil or assure us that the same problem would not re-occur, it was decided to replace Redi-earth® with soil mixtures from other

companies. Hence, an experiment was conducted to test the suitability of different soil mixtures for chickpea donor plant growth.

The following substrates were tested:

- Sunshine Mix® No 1 – 5
- Sunshine Bark® Mix SB 100 – 500
- Dutch Growers® Mix
- Sunshine Bark® Mix SB 400 + Perlite® + Vermiculite® + Lime
- ½ (Sunshine Bark® Mix SB 400 + Perlite® + Vermiculite + Lime) + ½ Sunshine Germination Mix® No. 3 with a final pH of 6.5 – 7.5

Results showed that a combination of Sunshine Bark® Mix SB 400, Perlite®, Vermiculite®, lime and Sunshine Mix No. 3 provided the best growing conditions for chickpea. Mix SB400 is a medium textured, well draining general purpose soil mix consisting of about 40% bark, 50% peat, 10% Perlite®, lime, gypsum, starter fertilizer and a wetting agent. Since this mixture tended to dry out fairly quickly, Vermiculite® and Sunshine Germination Mix® No. 3 was added in order to improve soil moisture retention. As a result, chickpea donor plant growth has considerably improved and 'dried up plants' are no longer a problem.

Light Intensity and Spectrum

Generally, donor plants grew much better in the greenhouse than in the growth chamber, both in Canada as well as Australia probably due to the high light intensity, as well as much better light spectrum from the natural light. However, growing plants in the greenhouse resulted in problems with pests and high temperatures (see Greenhouse section for further discussion). Hence, growth chambers with different light intensities and light spectrums were investigated previously. However, the results were fairly disappointing with higher light intensity chambers not resulting in better donor plant growth. Therefore, an investigation was undertaken to determine the cause for the lack of response and is discussed in the following section.

Growth Temperature

Initially, plants were grown at a temperature of 24/20° C day/night regime using a 16-hour photoperiod and a light intensity of 350 – 400 μEm^2 in order to improve germination. After 2 weeks, donor plants were transferred to a chamber with higher light intensity of about 400-600 μEm^2 and a temperature regime of 20/15° C day/night, respectively.

Depending on the type of chamber used, the plants looked either normal (chamber with high-pressure sodium lights mounted at the ceiling) or stressed (double-rows of fluorescent lights). Hence, tests were done to determine the cause of this stress. Temperature and light measurements were taken at pot level and at the top of the plants in every chamber. Results showed that in the high light intensity chamber, the actual temperature during the day at pot or plant level was at least 10°C higher

than set on the chamber display due to the high heat output from the ballast. Therefore, the temperature needed to be lowered during the day to about 12 °C in order to obtain the correct temperature of 20 °C.

Greenhouse

Renting space is considerably cheaper in the greenhouse ($1 per square foot per month including supplies) than in the Phytotron ($5.80 – 7.75 per square foot per month excluding supplies such as soil etc). Hence, using the greenhouse instead of growth chambers would considerably reduce costs for producing doubled-haploid plants. In addition, donor plant growth was generally much better in the greenhouse than in the growth chamber, especially in Australia. In Canada, donor plant growth in the greenhouse was much more variable and an investigation was undertaken to determine if the greenhouse was a viable alternative to the more expensive growth chambers.

Plants were initially grown in the greenhouse in section E. This section is very large and contains numerous species in different stages of growth. Initially, chickpea grew very well in this section until a batch of beans adjacent to the chickpea started to mature. As a result, pests such as thrips and white flies migrated from the beans to the chickpea causing some direct damage as well as subsequently higher contamination rates due to the thrips burrowing into the buds. In order to prevent such migration, the donor plants were moved to section D. This section contains fewer benches and species and hence, pests can be better monitored.

Besides pests, another problem in our greenhouse was the temperature fluctuations during the season. For example, the swamp coolers are started once the chance of frost is diminished i.e. they are usually turned on in June. However, temperatures soared in May of last year reaching temperatures in the greenhouse of up to 36°C, effectively killing all pollen in the donor plants.

The third problem with growing plants in the greenhouse was the short day length and low light intensity during the winter months. Material grown during the months of November to February were generally poorer in quality than material grown at other times. The additional high sodium lights mounted on the greenhouse ceiling did not provide enough light to compensate for the shorter day length.

In conclusion, the donor plants grown in the Australian greenhouses provided excellent material for doubled-haploid production with much higher numbers of inducible microspores, whereas the plants grown in the Saskatoon greenhouses were too variable to be reliable sources for doubled-haploid production. However, plants grown from February to April and from June to November provided better microspores at considerably lower costs if the temperature and pests could be controlled than material grown in the growth chamber.

Genotypes

Embryogenic and androgenic response as well as regeneration capacity varies with genotype and is under genetic control (Larson *et al* 1991, Powell 1990, Sonnino *et al* 1989). Hence, having genotypes that are known to be embryogenic might speed up our protocol development. Previously, Australian cultivars, Bumper, WACPE 2095, Amethyst and Narayen, were determined to be androgenic with Narayen generally having higher induction rates.

Subsequently, several Canadian genotypes and breeding lines were also screened with our improved protocols and CDC Cabri was also found to be highly androgenetic. Therefore, subsequent experiments were conducted with Narayen and/or CDC Cabri. Both genotypes preferred heat as pre-treatment.

3.2. Pre-treatment

Smykal and Pechan (2000) reported that heat shock proteins were activated within 30 minutes of heat treatment, reaching maximum levels after 6-8 hours in *Brassica* but these rates varied considerably between species. Both the absolute temperature as well as the length of the heat pre-treatment affected microspore induction rates. Previously, heat was also found to be an effective pre-treatment for switching microspores from the gametophytic to the sporophytic pathway (see Diagram 2 Chapter 5). However, since our results were quite variable, the different components of the pre-treatment were also investigated.

(1) Incubator

Heat pre-treatments were conducted in either of 2 incubators (FinePCR® Hybridization Incubator Combi H12) in the Pulse Molecular Laboratory. The incubators were the same models, purchased at the same time and the setting was identical as well. Whichever of the 2 incubators was free, was used for the pre-treatments. However, we noticed that the results obtained varied with the incubator used. Hence, tests were conducted to determine if there was a difference between the two incubators. A temperature probe was inserted in the incubator and the temperature was monitored continuously during the pre-treatment of the buds. Results showed that one of the incubators had an actual temperature of 36 ° C instead of 32.5 ° C even though the temperature setting was indeed correct. Chickpea microspores that were expose to 36 °C did not survive (DAPI staining showed no nuclei) indicating that the critical temperature is between 34 – 35 °C. Therefore, all subsequent pre-treatments were conducted in the incubator at 32.5 °C (right hand chamber, incubator #1).

(2) Temperature and length of treatment

An experiment was conducted testing different temperatures (27, 30 or 32.5 °C), different length of heat pre-treatment (8, 12, 16, 20, 22, or 24 hours) as well as two cultivars (Narayen and CDC Cabri). Based on the number of induced microspores obtained in the experiments, it seemed that 16 hours at 32.5°C was optimal for CDC Cabri. Narayen responded to all three temperatures as long as the treatment lasted longer at the lower temperatures e.g. 24 hours at 27 °C.

(3) Harvest number

Usually, buds were picked from the donor plants three times with the first 'harvest' about 4-5 weeks after seeding, 'harvest' No. 2 during week 6 and 'harvest' No. 3 during week 7. The number of buds obtained from the first 'harvest' was generally considerably lower and it was highest from the third 'harvest'. Based on the number of induced microspores, the third 'harvest' resulted in higher induction rates than the first. A subsequent experiment also tested 'harvest' No. 4 and found it equally responsive.

However, results showed that there were differences between batches i.e. sets of plants seeded at the same time. Some batches gave consistently good results, whereas microspores from other batches were unresponsive even though the batches were planted only one week apart and the same growing conditions were used i.e. same genotype, soil mix, fertilizer and watering regime, and growth chambers. All donor plants were healthy and showed excellent root and shoot growth. Hence, at this point, it is still not clear why these considerable variations occur. An experiment was set up to continuously monitor soil moisture and to determine if small changes in this factor had an influence on microspore responsiveness.

3.3. Sterilization Method

Our laboratory was flooded about a year ago due to a problem in the adjacent malting barley lab. As a result of this, major repair work was undertaken in the laboratory as well as in the entire Agriculture building. In the weeks following the repair work, we noticed a substantial increase in contamination rates. However, the problem seemed to be mostly caused by bacterial contamination rather than the expected increases in mold due to the excessive moisture in the laboratory as well as in the building walls and ceilings. In addition, the antibiotic cefotaxime that had been routinely used in our media was no longer effective in controlling bacterial contamination. Hence, further improvements in our sterilization technique as well as a major laboratory clean up was required.

Test plates were placed throughout the laboratory and on the laminar flow benches in order to determine the source as well as type of contamination. Results showed

that the laminar flow benches provided a sterile environment. However, the test plates located directly underneath the air vent near the entrance of the lab showed considerable amounts of bacterial contamination. Non-sterile buds were usually picked at the table adjacent to this air vent. Hence, this was most likely a major cause for contamination of our plant material and subsequent sterilization techniques were unsuccessful in removing these bacteria. As a result, all buds are now picked in the laminar flow bench and a thorough clean up of the laboratory was performed using bleach and Lysol®. A weekly lab clean up including washing floors and sterilizing frequently touched surfaces was also instigated. In addition, cleaning and sterilizing agents are frequently varied in order to prevent bacteria from becoming resistant. In addition, the different components of our sterilization technique were also scrutinized:

(1) Testing antibiotics:

Since cefotaxime was no longer effective in eliminating bacterial contamination, a number of antibiotics were tested in order to determine:

(a) effectiveness of antibiotic in eliminating bacterial contamination
(b) effect on microspore viability

The following antibiotics were tested using microspores obtained from CDC Cabri:

- Gentamycin: 50 mg/L
- Puromycin: 50 mg/L
- Rifampicin: 50, 100 or 150 mg/L
- Hygromycin: 50 mg/L
- Vancomycin: 50 or 100 mg/L
- Methicillin: 10 mg/L
- Carbenicillin: 50, 100, 150 mg/L
- Kathon®: 0.5, 1.0, 1.5, 2.0 mg/L

Results showed that even though the antibiotics were mostly effective in eliminating bacterial contamination, they unfortunately had a negative effect on microspore viability with the exception of Carbenicillin.

(2) Testing sterilizing agents:

- Bleach concentration: 10, 15, 20, 25 or 30% solutions
- Length of bleach treatment: 10, 15, 25, or 30 minutes
- Buffered hypochlorite solution (see below)
- Ostro-san® 800ppm
- Dettol® 25 ml/L
- Sporicine®
- Sodium dichloro isocyanurate® 2.5g/L
- Salicyclhydroxamic acid (SHAM 100 mg/L)

- Lysol® spray
- 70% Ethanol

Buds from CDC Cabri were sterilized using these solutions, then plated whole and crushed on test plates and incubated at 24°C in the dark for 1 - 2 weeks. Results showed that the buffered hypochlorite solution was the most effective sterilizing agent.

The buffered hypochloride solution is prepared as follows:
2 ml of a 10 % K_2HPO_4 solution
+ 2 ml of bleach (Javex®) containing app. 5% sodium hypochlorite
+ 96 ml of distilled H_2O
+ 2-3 drops of Tween® (detergent)

(3) Fungicides:

- Benomyl®: 5, 10, 15 or 20 mg/L
- Benzoate®: 0.5, 1.0, 1.5 or 2.0 mg/L

Mold is usually the most frequent contaminant in tissue culture. Hence, 2 fungicides were tested in order to determine if they could be used to eliminate mold in microspore cultures. However, results showed that they were ineffective in controlling mold and were also detrimental to microspore viability at all concentrations tested.

(4) Testing sterilizing techniques:

- Tea strainer ('eggs')
- Shaker
- Stirring bars and plates
- Ultrasonic water bath
- Vacuum infiltration

The surface of chickpea buds is densely covered with trichomes or 'hairs' (see Fig. 2 below) which prevents sterilizing solutions from penetrating the actual bud surface and thus killing bacteria and molds. In addition, buds also float on the surface of sterilizing solutions. Hence, an experiment was conducted to test the effectiveness of five different sterilization techniques. The following set ups were tested:

- Buds were placed into tea strainers and the entire strainer submerged in the bleach solution
- Buds were placed into sterile Erlenmeyer® baffle flasks, bleach solution was added, and Erlenmeyer flasks were placed on a shaker rotating at 150 RPM.
- Buds were placed into sterile beakers containing bleach and a stirring bar. The beaker was then placed on a magnetic stirring plate at medium speed.

- Buds were placed into 50ml sterile centrifuge tubes filled with bleach solution. The centrifuge tubes were then submerged in an ultrasonic water bath and sonicated for 15 to 30 minutes.
- Buds were placed into a sterile vacuum flask in a buffered hypochlorite solution and the flask was connected to another vacuum flask (to maintain sterility) and then to a vacuum pump for 20-30 min.

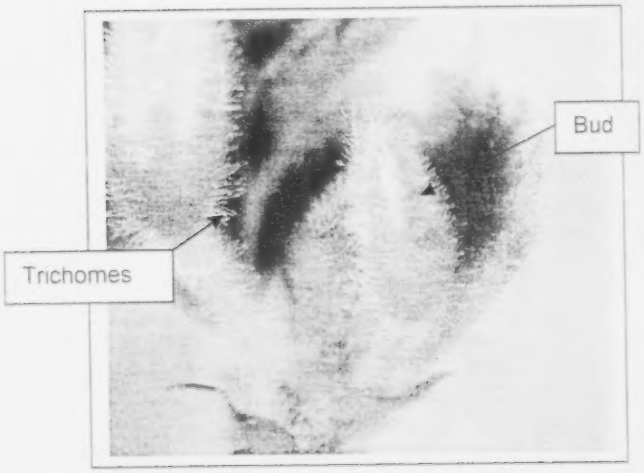


Fig. 2. Chickpea buds with trichomes

Buds from CDC Cabri were sterilized using the techniques described above. After rinsing with sterile distilled water, they were placed whole and crushed on indicator plates, incubated at 24°C in the dark for 1-2 weeks.

Results showed that vacuum filtration using a buffered bleach solution was the most effective and reliable method for sterilizing chickpea buds and since then, has been used as a routine method for sterilization (see Protocol Part II for details).

3.4. Culture Medium

Our induction medium ChM1 (Table 1) was developed in previous experiments. The composition was based on Murashige and Skoog's (1962) medium (MS) but has a lower ammonium nitrate (NH_4NO_3), higher iron (NaFeEDTA), zinc ($ZnSO_4$ 7 H_2O) and copper ($CuSO_4$ 5H_2O) concentration. Other modifications include different vitamin concentrations, a much higher sucrose content and added casein hydrolysate, an amino acid mixture instead of glycine. It also has plant growth regulators added not present in MS medium. This medium was found to be effective for induction of microspores from different genotypes both in Australia and Canada. However, efforts continued to improve this medium by varying macro and micro-nutrients, vitamins, organic nitrogen sources, plant growth regulators and other components. The different media tested are listed in Table 2.

Table 1. Composition of induction medium ChM1

Macro in mg/L	MS Medium	ChM1 Medium
NH_4NO_3	1,650 mg	**1,000 mg**
KNO_3	1.900 mg	1.900 mg
$CaCl_2$ $2H_2O$	440 mg	440 mg
$MgSO_4$ $7H_2O$	370 mg	370 mg
KH_2PO_4	170 mg	170 mg
Micro in mg/L		
NaFeEDTA	27.80 mg	**40.00 mg**
KI	0.83 mg	0.83 mg
H_3BO_3	6.20 mg	6.20 mg
$MnSO_4$ H_2O	16.91 mg	16.91 mg
$ZnSO_4$ $7\ H_2O$	8.60 mg	**10.00 mg**
Na_2MoO_4 $2H_2O$	0.25 mg	0.25 mg
$CuSO_4$ $5H_2O$	0.025 mg	**0.05 mg**
$CoCl_2$ $6H_2O$	0.025 mg	0.025 mg
Vitamins		
Myo-Inositol	100.00 mg	100.00 mg
Pyridoxine HCl	0.50 mg	**0.50 mg**
Thiamine HCl	0.10 mg	**0.50 mg**
Nicotinic acid	0.50 mg	**5.00 mg**
Amino Acids		
Casein hydrolysate		**200.00 mg**
Glycine	2.00 mg	
Carbohydrates		
Sucrose g/L	30.00 g	**90.00 g**
Plant Growth Reg.		
2,4-D mg/L		1.00 mg
Picloram mg/L		0.25 mg
BAP mg/L		0.10 mg
pH	5.8	5.8

Table 2. Variation in ChM1 composition

Medium Code	Component varied
ChM 2	34 mg/L PAA instead of 2,4-D
ChM 3	8 mg/L GA_3 added
ChM 4	1.58 mg/L Violuric acid added
ChM 5	28 mg/L Histidine added
ChM 6	3.00 mg/L 2,4-D added
ChM 7	2.00 mg/L 2,4-D added
ChM 8	1.500 mg/L 2,4-D added
ChM 9	200 mg/L glutamine added instead of casein hydrolysate
ChM 10	2.5g KNO_3 added; NH_4NO_3 omitted; 500 mg/L casein hydrolysate
ChM 11	BAP omitted
ChM 12	600 mg/L $CaCl_2 \cdot 2H_2O$
ChM 13	2.5 g/L KNO_3; NH_4NO_3 omitted
ChM 14	50 mg/L $ZnSO_4 \cdot 7\ H_2O$; 0.12 mg/L $CuSO_4 \cdot 5H_2O$; 1 g/L casein hydrolysate
ChM 15	2.5g/L KNO_3; NH_4NO_3 omitted; 600 mg/L $CaCl_2 \cdot 2H_2O$; 0.75 mg/L $CuSO_4 \cdot 5H_2O$; 500 mg/L casein hydrolysate; 500 mg/L glutamine; 50 mg/L sucrose; 0.75 mg/L 2,4-D
ChM 16	2.5g/L KNO_3; NH_4NO_3 omitted; 600 mg/L $CaCl_2 \cdot 2H_2O$; 0.75 mg/L $CuSO_4 \cdot 5H_2O$;
ChM 17	43.24 g/L glucose added; 5.4 g/L fructose added; sucrose omitted
ChM 18	24.32 g/L glucose added; 24.32 g/L fructose added; sucrose omitted
ChM 19	32.43 g/L glucose added; 16.21 g/L fructose added; sucrose omitted
ChM 20	10 mg/L NaFeEDTA; 50 mg/L 2,4-D added
ChM 21	0.5 mg/L Pyridoxine added; Thiamine omitted; 5 mg/L Nicotinic acid added; 50 mg/L Brassinolide added
ChM 22	0.5 mg/L Pyridoxine added; Thiamine omitted; 5 mg/L Nicotinic acid added; 200 mg/L Spermidine added
ChM 23	0.5 mg/L Pyridoxine added; Thiamine omitted; 5 mg/L Nicotinic acid added;
ChM 24	0.5 mg/L Pyridoxine added; Thiamine omitted; 5 mg/L Nicotinic acid added;
ChM 25	0.5 mg/L Pyridoxine added; Thiamine omitted; 2 mg/L Nicotinic acid added;
ChM 26	10 mg/L NaFeEDTA added; 50 mg/L Brassinolide added
ChM 27	10 mg/L NaFeEDTA added; 0.5 mg/L Pyridoxine added; Thiamine omitted; 2 mg/L Nicotinic acid added; 50 mg/L Brassinolide added
ChM 28	10 mg/L NaFeEDTA added; 75 mg/L Spermidine added
ChM 29	10 mg/L NaFeEDTA added; 0.5 mg/L Pyridoxine added; Thiamine omitted; 2 mg/L Nicotinic acid added; 50 mg/L Brassinolide added; 75 mg/L Spermidine added

ChM 30	10 mg/L NaFeEDTA added; 0.5 mg/L Pyridoxine added; Thiamine omitted; 2 mg/L Nicotinic acid added; 100 ml/L coconut water added; 4 mg/L Calcium Panthenotate added; 50 mg/L Brassinolide added; 75 mg/L Spermidine added
ChM 31	10 mg/L NaFeEDTA added;
ChM 32	11 mg/L Nicotinic acid added
ChM 33	4 mg/L Calcium Panthenotate added
ChM 34	50 mg/L Brassinolide added
ChM 35	100 ml/L coconut water added; 100 mg/L Spermidine added
ChM 37	20 mg/L Nicotinic acid added
ChM 38	17.4 mg/L Arginine added1.12 mg/L 4-CPA added
ChM 40	500 mg/L NH_4NO_3; 950 mg/L KNO_3
ChM1-60	60 g/L sucrose added
ChM1-30	30 g/L sucrose added
ChM1-L2	L2 macro, micro and vitamins
ChM1-NLN	NLN macro, micro and vitamins

Results showed that none of the media tested was an improvement to ChM1. For example, ChM1 was compared with ChM10 as well as ChM12 or 13 using both CDC Cabri and Narayen. ChM10 was either filter-sterilized (F) or autoclaved (A). Table 3 lists the number of symmetrical bi-nucleate and multi-nucleate microspores obtained in this experiment. For both cultivars, ChM1 was clearly better for inducing symmetrical microspore division. Autoclaving the medium resulted in a reduction of induced microspores probably due to hydrolysation of sucrose during autoclaving.

Our collaborator, Dr. Croser found that reducing ammonium and nitrate in the ChM1 medium but keeping the relative ratio of both compounds the same improved induction in her experiments. Hence, we conducted a similar experiment with our material. Preliminary results indicate that both media are effective in inducing microspore induction. However, the effect on embryo development has not yet been determined.

Table 3. Experiment comparing ChM1 with ChM10, 12 and 13 (F = filter-sterilized medium; A = autoclaved medium)

Cultivar	Medium	1 nucleus	Bi (2n)	Tri (3n)	4 n	5 n	6 n	8 n
Narayen	**ChM1**	**634**	**1106**	**36**	**16**	**4**		
	ChM10F	238	456	26	8	2		
	ChM12	100	86		1			
Cabri	**ChM1**	**742**	**1556**	**82**	**28**	**8**	**2**	**2**
	ChM10F	160	296	22	12	10	2	
	ChM10A	72	152	28	4	2		
	ChM13	370	446	16	8			

3.6. Embryo Development

Previously, published protocols for chickpea regeneration were tested on induced microspore cultures. However, even though large numbers of pro-embryos were present in the cultures (see Figs 6 and 7 in photo file), no plant regeneration could be obtained and pro-embryos continued to callus. **Diagram 1** below shows that the initiation of embryogenesis in legumes generally requires a relatively high amount of auxin (e.g. 2,4-D, Picloram, 4-CPA, IAA) and a relatively lower amount of cytokinin (e.g. BAP, zeatin). However, once embryogenesis has been initiated, the mixture needs to shift towards lower auxin and higher cytokinin content for shoot regeneration. The absolute amount of each growth regulator depends on each species and even varies with genotype as well as other culture medium components. For example, Kathari *et al* (2004) found that the auxin NAA could be replaced in the medium by doubling the KNO_3 or quadrupling the FeEDTA amount in the medium. Therefore, the development of a regeneration medium needs to take the entire medium composition into account.

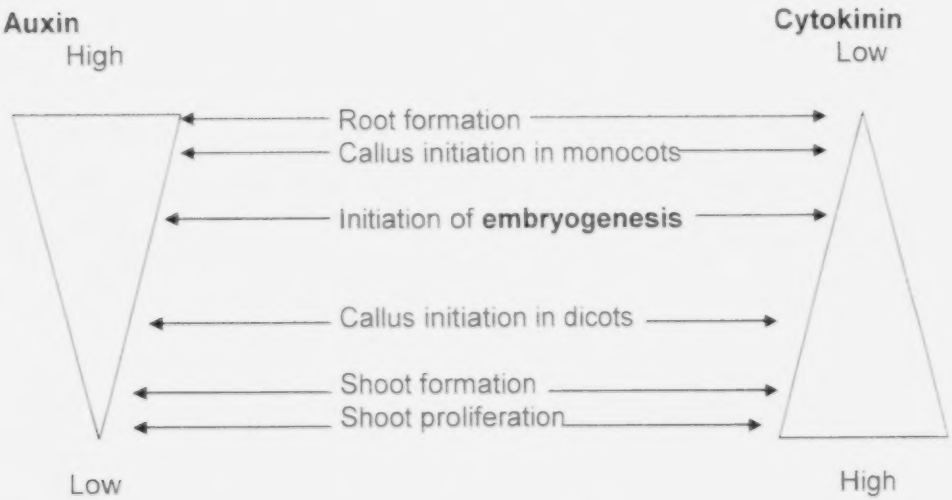


Diagram 1. Simplified diagram of auxin – cytokinin interaction in plant development (Source: George (1993) Plant propagation by tissue culture. Part 1 The Technology)

The major focus of our experiments continued to be the development of a regeneration protocol. In our current protocol, 7 – 21 days after induction, microspores suspended in liquid ChM1 were transferred to centrifuge tubes and centrifuged at 1000 RPM for 3 minutes. The supernatant was then discarded and the pellet re-suspended in Adkins *et al* (1995) medium (unless otherwise noted). The microspores were then pipetted onto Gelman grid filters placed on different embryo development media (EDM). Our experiments tested 4 aspects of the regeneration protocol: (1) the medium, (2) the gaseous atmosphere, (3) separation of different stages by sieving and (4) centrifugation step.

(1) Embryo Development Media

The four base media used in our experiments are listed in Table 4. The L2-D medium is based on a medium developed by Phillips and Collins (1979) for regeneration of legumes. The G1-P1 medium was developed by Geerts *et al* (1999) for *Phaseolus* and is based on Phillips *et al* (1982). Medium 13C is a derivative of Mallikarjuna's medium (1999) for regeneration of interspecific chickpea hybrids. The composition of the base medium was varied and the resulting different embryo development media are listed in Table 5. In order to develop a regeneration medium, we looked at the different aspects that might affect callusing:

- Base medium composition (macro- and micro-nutrients)

Chauhan and Kothari (2004) found that nutrient requirements varied with induction, subculture and regeneration of barley cultures. For example, reducing the amount of NH_4NO_3 in the medium by half and increasing the amount of KH_2PO_4 five times considerably improved subsequent regeneration. Therefore, four base media (Table 4) were tested with various amounts of NH_4NO_3 (from 0 – 1000 mg/L) and KH_2PO_4 (166 – 405 mg/L) as well as many other minerals (e.g. Mg from 179 – 435 mg/L, FeEDTA from 10 – 35 mg/L or Mn from 10 – 22 mg/L). Neither of the modifications tested improved regeneration.

- Auxins

Auxins are necessary for plant cell growth since they are involved in cell division. In our current protocol, a combination of 2,4-D and picloram was found to be necessary for microspore induction. However, 2,4-D can be very persistent in plant tissue but any attempt at replacing 2,4-D with other auxins in previous experiments resulted in either complete failure or very low rates of induction. Therefore, embryo development media were tested that had no (e.g. L2-D) or very low (13C) amounts of auxin added. However, results showed that absence of auxin by itself did not prevent callusing.

- Cytokinins

Regarding cytokinins, both the type and concentration of this growth regulator influences regeneration. For example, wild *Cicer* species (e.g. *C. bijugum* and *C. judaicum*) require different types of cytokinins for regeneration (BAP or zeatin). Hence, media were made using different concentrations of BAP, zeatin or zeatin riboside as well as coconut milk was added, which contains natural cytokinins. Results showed that neither cytokinin type nor concentration made any difference for regeneration.

Table 4. Medium composition for embryo development media

Macro in mg/L	**L2-D**	**G1-P1**	**13C**	**EDM56A**
NH_4NO_3	1,000	400	1,000	
KNO_3	2,100	2,500	1,000	1,950
$CaCl_2$ $2H_2O$	600	600	330	500
$Ca_2(NO_3)_2$ $4H_2O$			500	
$MgSO_4$ $7H_2O$	435	250	179	185
KH_2PO_4	325	-	170	166
NaH_2PO_4 H_2O	85	150		
$(NH_4)_2$ SO_4		134		166
Micro in mg/L				
NaFeEDTA	25.00	35	35	27.85
KI	1.00	0.75	0.83	0.83
H_3BO_3	5.00	3.00	6.20	6.20
$MnSO_4$ H_2O	15.00	10.00	22.00	16.91
$ZnSO_4$ $7H_2O$	5.00	2.00	8.60	8.60
Na_2MoO_4 $2H_2O$	0.40	0.25	0.25	0.25
$CuSO_4$ $5H_2O$	0.10	0.025	0.025	0.025
$CoCl_2$ $6H_2O$	0.10	0.025	0.025	0.025
Vitamins in mg/L				
Myo-Inositol	250.00	100	100	100
Pyridoxine HCl	0.50	0.50	1.00	0.50
Thiamine HCl	2.00	1.00	10.00	0.50
Nicotinic acid		5.00	1.00	5.00
Amino Acids in mg/L				
Glutamine	500	1000		2,000
Casein hydrolysate		1000	200	
Aspartic acid				150
Arginine				100
Leucine				50
Lysine				50
Phenylalanine				50
Serine				50
Proline				50
MES				2,000
Carbohydrates				
Sucrose g/L	25.00	30.00	30.00	40.00
Glucose g/L				40.00
Plant Growth Reg.				
BAP	1.00		1.00	
IAA			0.10	
Phytagel in g/L	2.75	2.75	2.75	2.75
PH	5.8	5.8	5.6	5.8

Table 5. Medium composition for embryo development media

EDM	Composition
L2-A	Same as L2-D but no amino acids
L2-B	Same as L2-D but no amino acids + 5 mg/L 2-HNA + 1 mg/L Thiamine
L2-C	Same as L2-D but no other amino acids than 1000 mg/L glutamic acid
L2-E	Same as L2-D but no amino acids + 75 g/L sucrose
ED-F	MS macro + micro + B5 vitamins + 20 g/L sucrose + 1.126 mg/L BAP
EDM-28	Same as 13C except 27.85 mg/L FeNaEDTA; 20 g/L sucrose; 20 g/L glucose; 1 mg/L GA_3; IAA omitted
EDM-29	Same as EDM-28 except BAP replaced by 1 mg/L zeatin
EDM-29B	Same as EDM-29 but casein hydrolysate and IAA omitted
EDM-34	Same as EDM-29 but casein hydrolysate omitted
EDM-38	Same as EDM-28 but casein hydrolysate omitted + 0.5 mg/L zeatin + 0.5 mg/L zeatin riboside + 0.125 mg/L IAA
EDM-40	MS macro and micro + B5 vitamins + 2.253 mg/L BAP + 10.56 mg/L ABA + 20 g/L sucrose + 3.0 g/L phytagel
EDM-41	MS macro and micro + B5 vitamins + 2.253 mg/L BAP + 50 µM salicylic acid + 3.0 g/L phytagel + 50 µM salicylic acid
EDM-42	MS macro and micro + B5 vitamins + 200 mg/L glutamic acid + 100 mg/L arginine + 100 mg/L aspartic acid + 0.5 mg/L zeatin + 1.0 mg/L GA_3 + 20 g/L sucrose + 3.0 g/L Phytagel
EDM-43	MS macro and micro + B5 vitamins + 200 mg/L glutamic acid + 100 mg/L arginine + 100 mg/L aspartic acid + 2.253 mg/L BAP + 20 g/L sucrose + 3.0 g/L Phytagel
EDM-44	MS macro and micro + B5 vitamins + 1 mg/L zeatin + 200 mg/L casein hydrolysate + 10 g/L sucrose + 20 g/L myo-inositol + 3.0 g/L Phytagel
EDM-45	MS macro and micro + B5 vitamins + 1.126 mg/L BAP + 10 g/L sucrose + 30 g/L myo-inositol + 3 g/L Phytagel
EDM-46	MS macro and micro + B5 vitamins + 1.126 mg/L BAP + 20 g/L sucrose + 21.3 g/L mannitol + 10 mg/L proline + 3 g/L Phytagel
EDM-47	MS macro and micro + B5 vitamins + 1.126 mg/L BAP + 10 g/L sucrose + 21.3 g/L sorbitol + 3 g/L Phytagel
EDM-48	MS macro and micro + B5 vitamins + 1.126 mg/L BAP + 20 g/L sucrose + 0.1 µM brassinolide
EDM-49	MS macro and micro + B5 vitamins + 1.126 mg/L BAP + 20 g/L sucrose + 3 g/L Phytagel
EDM-50	MS macro and micro + B5 vitamins + 1.126 mg/L BAP + 30 g/L maltose + 3 g/L Phytagel
EDM-56B	Same as EDM-56A + 1 mg/L zeatin
EDM-56C	Same as EDM-56A + 0.5 mg/L BAP + 0.5 mg/L GA_3
EDM-56D	Same as EDM-56A + 0.5 mg/L BAP + 1 mg/L IAA
EDM-57	Same as EDM-56A + 20 g/L sucrose + 20 g/L glucose
EDM-60	MS macro- and micro-nutrients + B5 vitamins + 2 mg/L BAP + 0.125 mg/L IBA + 30 g/L sucrose + 1.69 ml $AgNO_3$

CEDM-K	MS macro- and micro + B5 vitamins + 20 g/L sucrose + 2.253 mg/L BAP + 10.56 mg/L ABA
CEDM-T	½ MS macro and micro + 30 g/L sucrose + 1mg/L zeatin

- Other types of growth regulators

Another naturally occurring growth regulator is abscisic acid (ABA) that sometimes inhibits callus growth, promotes morphogenesis and is often necessary during embryo maturation. However, ABA had no effect on regeneration in our experiments.

Gibberelins (GA_3) are another class of growth substances that are involved in shoot formation but it can also promote callusing in combination with auxin and low cytokinin levels. However, using medium EDM-56C with a combination of 0.5 mg/L BAP and 0.5 mg/L GA_3 did not improve embryo development.

The effect of another group of growth regulators, the anti-gibberellins (e.g. chlormequat, ancymidol) has not been tested to date due to insufficient amounts of induced microspores. For example, anti-gibberellins stimulated embryogenesis in *Citrus* by inhibiting GA_3. Hence, the effect of anti-gibberellins on embryo development should be investigated.

- Vitamins

Similarly, the concentration and combination of vitamins was varied and no improvement was found.

- Carbohydrate source and osmotic potential of the medium

The type and concentration of carbohydrates in the medium can have a major effect on regeneration by either acting directly as an energy source or indirectly by determining the osmotic potential of the medium. Even though all medium components contribute to the osmotic potential, the carbohydrates usually have a major affect due to the large amounts added. From the literature, it is indicated that a medium with a high osmotic potential induces embryogenesis and shoot regeneration, whereas mediums with low osmotic potential favors callus growth. Hence, we tested several media with different high (negative) osmotic potential. For example, carbohydrate concentrations were varied using different concentrations of sucrose (10 – 75g/L) as well as different combinations of carbohydrate sources (glucose + fructose, maltose, mannitol, sorbitol or myo-inositol). However, none of these components improved regeneration.

Agar concentrations can also affect the osmotic potential of the medium and Matthias (1988) found that higher agar concentrations improved embryo development and conversion in *Brassica*. No such effect was found in our tests.

- Organic nitrogen sources

The absence or presence as well as the type and combination of organic nitrogen sources can affect regeneration. Casein hydrolysate (CH) is derived from milk protein and contains a wide variety of amino acids. However, the concentration of the different amino acids is usually not optimal for plant growth. Hence, a mixture of different amino acids in the relative amounts present in mature chickpea seeds was also tested. Again, no positive effect on plant regeneration was observed

The number of experiments that could be conducted was limited by the amount of induced microspores available for testing especially since a high microspore density was also necessary for regeneration. Therefore, not all concentrations and combinations could be tested. However, regardless of the treatment applied pro-embryos generally callused and regeneration could not be obtained. Therefore, the problem of callusing might be due to other factors that had not yet been addressed in our embryo medium experiments. The following factors were considered.

(2) Ethylene

Ethylene is another plant growth regulator that is affecting growth through its presence in the gaseous atmosphere of the plates in very low concentrations. This plant growth regulator is naturally released from growing plant tissue but concentrations can also increase by adding methionine, aspartic acid or cysteine (casein hydrolysate) as well as mannitol to the medium. Furthermore, addition of auxin to the medium can also result in increased ethylene production. In contrast, the micro-nutrient cobalt inhibits ethylene production if present in higher concentrations as does gibberellic acid, EDTA (see Fe) or cytokinins. It seems that a critical concentration of ethylene is often necessary at which morphogenesis is stimulated whereas lower or higher concentrations can be inhibiting or be ineffective (George 1993). The size of the culture plate as well as type of material used for closure (e.g. Parafilm®) of the vessels also affects the concentration of ethylene in the atmosphere.

An experiment was conducted using three types of culture plates, the small 4 cm diameter Falcon® plate, a 5 cm diameter medium size Petri® plate and a 9 cm diameter deep dish Petri® plate. In addition, two types of enclosures, either Parafilm® or Saran® wrap (the latter is supposed to allow better air exchange) were also tested. Results showed that embryos callused under all conditions.

Furthermore, induced microspores were also placed on medium EDM-60, which contained silver nitrate, and medium EDM-41, which contained salicylic acid. Both compounds are supposed to inhibit ethylene. Plants could not be regenerated on either medium. Therefore, ethylene does not seem to be the causal agent for callusing of microspore derived pro-embryos.

(3) Sieving

Dr. Heberle-Bors suggested to our Australian colleague Dr. Janine Croser, that concentrating induced microspores and removal of dead microspores might be a critical step in our protocol. Previously, we had tried to separate microspores into different fractions using density centrifugation. However, separation was poor and culturing the different fractions did not improve induction. Hence, a different approach was attempted by separating the different stages of microspores through filtration since the microspores differ in size. Mono-nucleate chickpea microspores are about 18 – 24 micron in size. Bi- and higher nucleate microspores are larger especially once microspore division takes place. Hence, separation of the different microspore fractions should be possible through sieving.

An experiment was conducted to determine if bi- or multi-nucleate could be separated from dead or mono-nucleate microspores. A series of sieves were tested with mesh sizes of 5, 10, 15, 25, or 35 micron. However, analysis of the filtrate after each step showed that even though microspores were clearly larger than 15 micron, they still passed through the 15 or 10 micron nylon filter mesh and only poor separation could be obtained. However, induced microspores could be collected on top of the 5 micron filter and separated from the dead microspores with few losses. Subsequently, the different fractions were collected and tested on different EDM media. In general, regeneration did not improve by culturing the different fractions. However, some cotyledonary/torpedo stage embryos were obtained from experiment CP020304:

Experiment CP020304

Microspores from cultivar Narayen were induced as usual using heat for 16 hours at 32.5°C. They were cultured in ChM1 for 2 weeks and good induction rates were obtained. Then, microspores were collected and transferred to a centrifuge tube and centrifuged for 3 minutes at 1000 RPM. The supernatant was removed and the microspores re-suspended in Mallikarjuna's (1999) liquid medium containing 1 mg/L zeatin and 0.25 mg/L IAA. They were then pipetted onto EDM-46 using a Gelman® grid filter and later transferred to medium 13C. Since the pro-embryos callused on these media, they were then transferred to G1-P1 medium without any growth regulators using 5 cm diameter Petri® plate. Some embryos regenerated to the cotyledonary stage on this plate 8 months after induction (see Diagram 2 Overview in chapter 5. Conclusion). Other microspore solutions were later placed on G1-P1 medium but regeneration could not be obtained using this medium by itself.

(4) Centrifugation

Generally, 1 to 3 weeks after induction, microspores were collected, transferred to a centrifuge tube and centrifuged for 3 minutes at 1000 RPM (see Protocol attached – Part II). The supernatant was then discarded, the microspores re-suspended in a small amount of Adkins' (1995) or Mallikarjuna's (1999) medium and transferred to

solid plates. However, regardless of the numerous different media or culture vessel used, pro-embryos callused and regeneration could not or only rarely be accomplished. Therefore, we reasoned that some step in our protocol might be inducing the embryos to callus. One such step could be the centrifugation of the induced microspores or pro-embryos before transfer to solid plates. It is possible that the centrifugation itself disturbs the polarity of the pro-embryos and causes the undifferentiated growth.

An experiment was conducted in which microspores were transferred to conical centrifuge tubes but left to settle for several hours without centrifugation. The supernatant was then removed as usual, replaced with Adkins (1995) medium and then pipetted onto solid plates. Preliminary results indicate that to date, these embryos have normal growth and have not yet shown signs of callusing.

4. Collaboration

This project was conducted in close collaboration with Dr. Janine Croser at CLIMA (Australia) who also worked on developing a chickpea microspore culture protocol. Information was exchanged frequently via e-mail, telephone conferences and visits. Here are some highlights from her experiments:

(1) For Narayen, 8 hours of heat pre-treatment at 30 °C was better than 27, 32.5, 35 or 37.5°C as well as a treatment of 16 hours at 30 °C
(2) Bud size, harvest number, pre-treatment conditions, density and medium requirements vary with each genotype
(3) For Narayen, a bud size of 2-3 mm is optimal.
(4) 'Harvest' number three gives better results than 'harvest' No. 1 or 2 i.e. older buds are more responsive than younger ones.
(5) Cultivar Sona requires cold pre-treatment whereas Narayen requires heat pre-treatment of at least 8 hours
(6) Regarding ChM1, a slightly higher pH of 6.0 was better for microspore viability
(7) There was no difference observed on microspore induction between shallow and deep dish Petri® dishes
(8) A sucrose concentration of 90g/L or 100 g/L was better than any other sucrose concentration or fructose, glucose or maltose in ChM1
(9) Varying 2,4-D concentrations showed that the control (1 mg/L) was better than 0, 10 or 100 mg/L
(10) Electroporation was not an effective pre-treatment for cultivar Bumper
(11) Generally, lower ammonium rates and a decreased ammonium to nitrate ratio is better for induction

Pro-embryos could also be induced in Australian experiments. However, regeneration of embryos into plants has also not yet been accomplished.

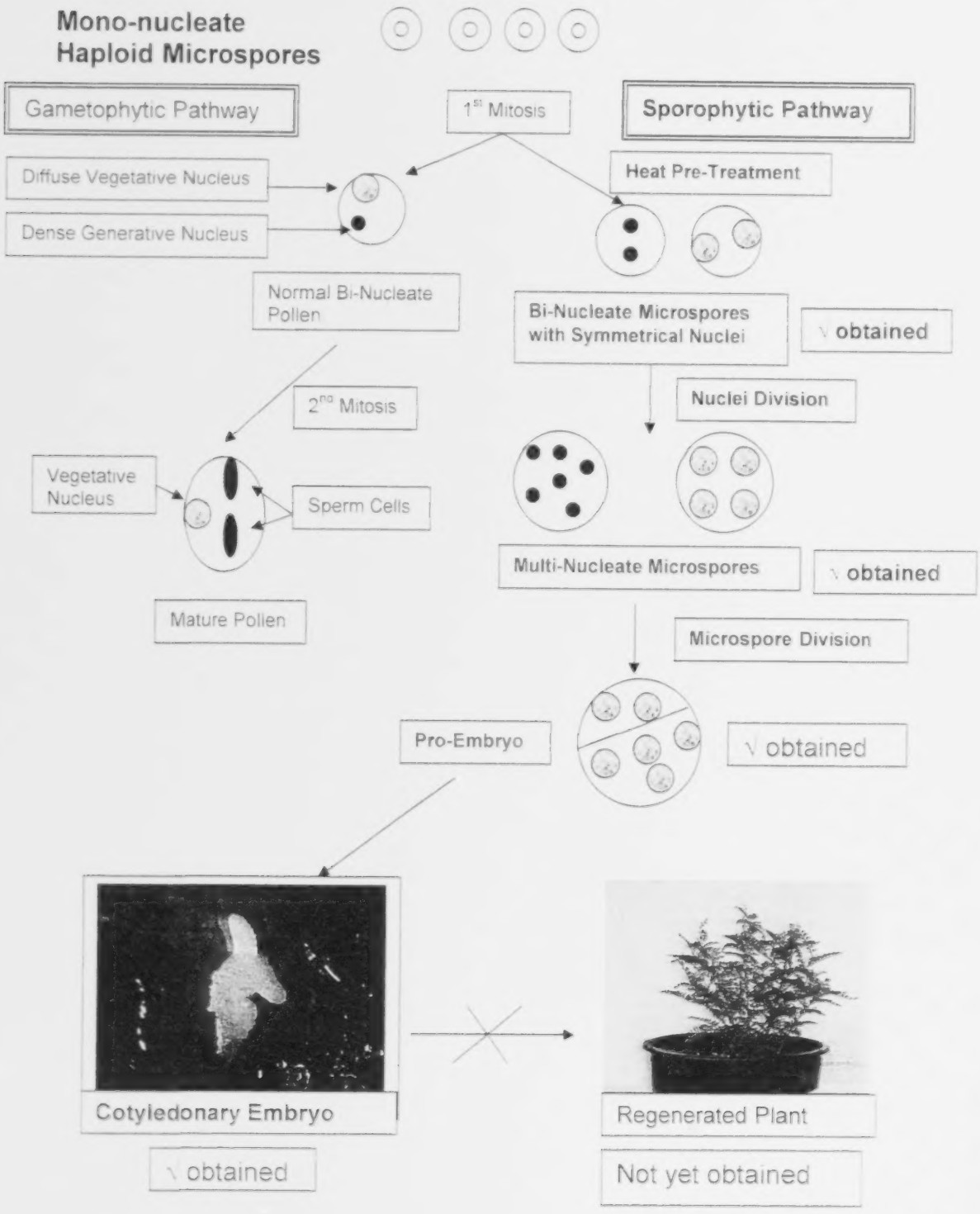
The Australian Grain Research and Development Corporation (GRDC) are currently considering a continuation of the CLIMA project for another two years. In addition, four more years of funding has also been requested for this project from the Australian Research Council.

5. Conclusion

Over the last three years, considerable progress has been made towards the development of a doubled-haploid protocol for chickpea. To date, a protocol has been developed jointly with Australian researchers in which chickpea microspores can be switched from the gametophytic to the sporophytic pathway and then be regenerated into embryos up to the torpedo/cotelydonary stage (see **Diagram 2** below or photos in Photo file). However, conversion of embryos into plants has not yet been accomplished.

In general, regeneration is often an impediment in legume tissue culture and conversion of embryos to plants is also problematic in chickpea somatic embryogenesis protocols. The low efficiency of regeneration has been attributed to poor histo-differentiation and abnormalities in maturation and germination. To date, successful doubled-haploid protocols for legumes have only been developed for pigeonpea (*Cajanus cajan* L.) and lucerne (*Medicago sativa* L.). Similarly to chickpea, soybean doubled-haploidy has also stalled at the regeneration stage. Therefore, more thorough and concentrated efforts are needed in order to address some of the basic problems concerning the various factors inducing regeneration in chickpea.

Diagram 2. Overview of Doubled-Haploid Production Stages in Chickpea



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F. Personnel paid by this project

Tim Dament: 12 months per year

G. Equipment

No equipment was purchased with this grant.

H. Project Developed Materials

Development of a doubled-haploid protocol is in progress. Posters were presented at the 5th European Conference on Grain Legumes in Dijon, France in 2004 and at Pulse Days in Saskatoon in 2005. A paper was submitted to Plant Cell Reports jointly with Dr. Croser in February 2005 on our chickpea doubled-haploid work. An invited review article about Doubled-Haploid Production in Legumes was written jointly with CLIMA and SARDI researchers and will be submitted to Plant Cell Reports in April of 2005.

I. Project Photos

Due to the size of the file, photos will be sent in a separate file.

J. Acknowledgments

Support of the Saskatchewan Agriculture Development Fund was acknowledged in all presentations in which this research was described.

J. Expense statement

Will be provided separately.

Microspore Culture Protocol For Chickpea (*Cicer arietinum* L.)

Developed
at the

Crop Development Centre
University of Saskatchewan

March 2005

A. Donor Plant Growth and Care

Excellent donor plant material is a prerequisite for microspore culture. For this reason, providing optimal growing conditions requires a good soil mixture, high light intensity, sufficient fertilizer and careful watering.

1. **Potting Mix**
   - Currently a mixture of ½ (Sunshine Bark Mix SB 400® + Perlite® + Vermiculite® + Lime) + ½ Sunshine Mix No. 3 is used.

   - This is an aggregate mix with a high air capacity, fast drainage and adequate water retention. It contains a mix of Bark, Canadian Sphagnum peat moss, Perlite®, major and minor nutrients, gypsum, dolomitic lime and a wetting agent. The ph is 6.5 – 7.5 on average.

2. **Preparation of Pots**

   - Use 11-liter pots. Place paper towel at the pot bottom to cover the holes. Fill the pots with the soil mixture and bump or lightly compress the soil in order to settle the soil evenly in the pots.
   - Water in ≅ 60 grams 14-14-14 nutricote™ granular fertilizer and ≅ 6 grams of nutritrace™ at initial filling of the 11 Litre pots and ensure that the soil is evenly wetted in the pot. Allow the filled pots to drain over night to prevent water saturation.

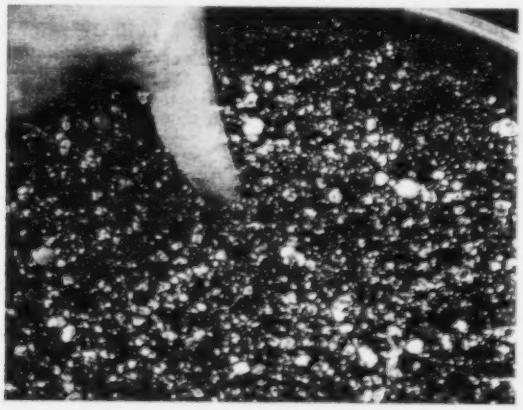
- Water the pots to near saturation and allow the them to drain overnight in order to obtain even distribution of moist, but not too wet soil conditions

3. Planting

- Plant 10 seeds (*over-seed if desired, and thin after 2 weeks*) in a circle in each pot. Place seeds about 2 cm beneath the soil surface and inoculate with rhizobium at this time. Cover the seeds with soil, but do not compress, this allows for good aeration.

- Label pots with genotype, batch number (i.e. Planting #. i.e. CP 23), date of planting, harvest number and chamber transfer regime.

- **Plants that will be used for seed increase**: Place about 35 grams of Nutricote® slow release granules in centre of the pot or lightly spread over entire soil surface after the seeds have been placed.



4. Growing Conditions

- Germination is initiated using a 24/20° C day/night regime with a 16-hour photoperiod of ~ 400 µE.

- Two weeks after seeding, the plants are transferred to a 20/15°C-day/night regime with a 16 hour photoperiod of ~ 430µE.

- If humidity levels can be controlled set levels to 45% during the day and 65% at night.

5. Plant Care

- *Water **carefully** and only as needed. Some pots dry out faster than others, therefore check each pot for "proper weight". ***DO NOT OVERWATER. Chickpeas are highly susceptible to root rot. The amount of water required varies with the age of the plants and the light intensity.****
- Check each pot to ensure similar moisture content. Occasionally, a pot may require supplemental watering.
- Plants grown for seed increase will need to be staked. Use 4 stakes per pot and fix cable ties around the plants for support.
- Plants are generally ready for the first harvest about 4-5 weeks after seeding, and is genotype dependant. It is best to harvest only twice, however a third harvest may be possible if the donor material is in good condition.

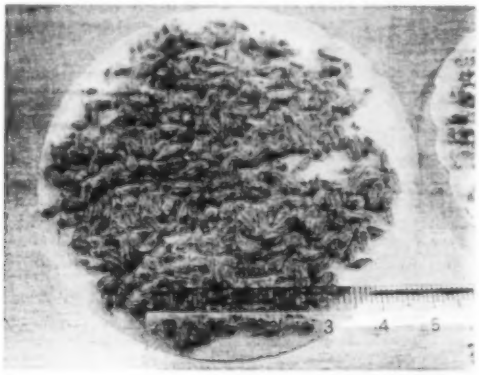
- If plants are to be kept for a length of time (i.e. seed increase). Fertilize with "flowering mix" or in house fertilizer in the growth facility, being careful not to expose foliage to fertilizer burning.

B. Bud Cluster Collection

1. **Equipment required**: plastic container with lid and moistened paper at the bottom, pen and tape.
2. Buds are picked weekly starting at 4-5 weeks after seeding.
3. Record: genotype, batch #, harvest #, seeding date and condition of donor plants (see appendix for experiment info sheet).
4. Pinch off the entire cluster containing buds about 3-5 mm in size.

C. Bud Selection

1. **Equipment required:** ultrafine tweezers, deep dish Petri® plate with moistened filter paper, and paper towels.
2. On laminar flow bench, pick buds with microspores at the uni-nucleate stage. Bud size is about 3-5 mm but may vary with genotype and harvest #. Anthers are usually greenish to slightly orange.
3. Place buds on filter paper in a deep dish Petri® plate.
4. Discard all buds that are too small (tetrad stage), too large (bi-nucleate) or are open. **This is important**!
5. Estimate the number of buds and record on experiment info sheet (attached in appendix).
6. Transfer buds to a sterile plate lined with sterile filter paper. Buds should be moist but not wet.



D. Pre-Treatment

1. Turn on incubator and set to 32.5C, at least 15 min. prior to use.
2. Put a note on the incubator to ensure it won't be turned off or the temperature won't be changed.
3. Place small Falcon Petri® dish containing a few millilitres of sterile distilled water into deep Petri® dish and wrap deep plate with Parafilm®. This will keep an elevated humidity level in the larger dish.
4. Record the starting time of the heat treatment (usually 4 pm) and remove buds after 16 hours (8 am).

E. Sterilization Procedure

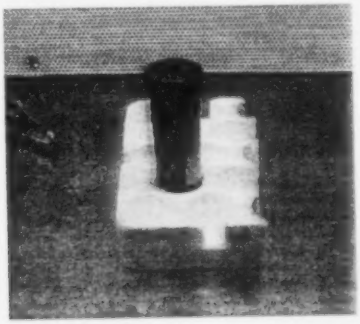
Note: all equipment and solutions used from this point on must be sterile and cold.

1. **Equipment required**: Buffered Hypochlorite solution + tween, tweezers with blunt tips, 1 L distilled water, small blender cups, 0.3M mannitol, ChM1, 2 nylon filters, 2 vacuum flasks, one 100 µm filter, one 35.5 µm filter nylon filter, one 5 µm nylon filter, Pasteur® and plastic pipettes, 50 and 15 ml centrifuge tubes, 100 µl pipettor, 1-100 µl pipette tips, 5 ml pipettor 100-1000 µl tips, cell counter, Carnoy® solution, deep dish Petri® plates, sterile paper filter for Petri® dish, 2 ml centrifuge tube for DAPI sample and a calculator
2. Place buds in vacuum flask. Add buffered hypochlorite solution + Tween®.
3. Stir for 20-minutes under vacuum. Drain bleach through sterile nylon filter sitting on top of vacuum flask.
4. Rinse with sterile distilled water (1 litre) to remove hypochlorite solution or until no bleach smell remains.

5. Place buds in vacuum flask. Add Buffered Hypochlorite solution + tween.
6. Stir for 20-minutes under vacuum. Drain bleach through sterile nylon filter sitting on top of vacuum flask.
7. Rinse with sterile distilled water (1 litre) to remove hypochlorite solution. (no bleach smell!).

F. Microspore Isolation Procedure

1. Transfer buds to a blender cup using blunt forceps.
2. Add a small amount of 0.3M mannitol (about 15 – 20 ml).
3. Macerate the buds with short bursts using a blender and tap the buds down in the blender cup between bursts.
4. Pour solution into 100 µm filter placed in the top of a glass beaker.
5. Transfer bud slurry back to blender cup with blunt tweezers.
6. Add more mannitol and repeat the procedure 2 - 4 until most of the microspores have been removed from the buds.
7. Transfer the microspore solution to a 50-ml centrifuge tube and centrifuge for 3 min. at 1000 RPM.



8. Remove the supernatant carefully without disturbing the pellet, using the 5-ml pipettor, be sure to remove any suspended cells above the pellet.
9. Resuspend the pellet in mannitol and gently drip onto the 35.5-micron filter placed on top of a beaker.
10. Rinse filter with mannitol until most microspores have been collected in the filtrate.
11. Collect the filtrate from the beaker using a 5 ml pipette and pipette the microspores gently onto a 5-micron nylon filter placed on top of a beaker.

12. Add more mannitol to rinse the filter and collect all microspores.

Always keep the microspores moist.

13. Allow several ml of mannitol to pass through the filter to remove any possible bacterial contamination.
14. Collect the filtered microspores from the filter and transfer to a 15 ml centrifuge tube, and dilute to 10 ml with mannitol.

15. Using a 100 µl pipette, add a small sample of the suspension to a haemocytometer. **Be sure to maintain asceptic technique** .
16. Take a sample (≈ 200µl) for DAPI staining and transfer to a 2-ml centrifuge tube. Centrifuge this sample and remove supernatant.
17. Add about 500 µL of Carnoy solution to the DAPI sample and store the sample in a fridge for about 24 hours (see notes for DAPI analysis).
18. Determine the number of microspores under a microscope. (haemocytometer)
19. Calculate the microspore density (see attached formula) and add an amount of ChM1 required to make a 250,000 or 500,000 microspore per ml solution.
20. Centrifuge again for 3 min at 1000 RPM.
21. Remove the supernatant without disturbing the pellet.
22. Re-suspend the microspores in the appropriate media and aliquot to desired medium.
23. Transfer the microspore solution to the appropriate plate depending on the cell density (well plate for 1 ml, small or deep dish Petri® plates for larger samples).
24. Transfer the plate to a thermal controlled incubator (dark, 24C).

G. Density Determination

1. Load the haemocytometer slide until the field is full (about 2 µl).
2. Place slide under the stereo light microscope using a 10x magnification.
3. Count all microspores within appropriate area.
4. If there is a lot of variation between the squares, count the microspores in 2 or 3 squares and average the value.
5. **Example**:

- If you have a 1 ml microspore solution and counted 236 microspores in one square then you would have:
  236 x 10,000 = 2,360,000 microspores per ml
  If you want a final density of 500,000 microspores per ml, then divide
  2,360,000 : 500,000 = 4.72 ml
  Therefore, you would add 3.72 ml of ChM1 to the 1-ml solution for a final volume of 4.72 ml.
- If your microspore pellet is quite large, dilute to 10 ml. Take your sample as above and calculate:
  236 x 10,000 = 2,360,000 microspores per ml x 10 ml = 23,600,000 microspores
  23,600,000 : 500,000 = 47.20 ml – 10 ml = add 37.20 ml to the 10 ml to a final volume of 47.20 ml.

H. DAPI Sample Preparation

1. Store Carnoy samples overnight in a fridge.
2. Remove the Carnoy and add 500µl of 70% ethanol, if samples cannot be processed after 24 hours. Continue to store in fridge.

3. Centrifuge sample for 2 min. at 4000 RPM.
4. Remove Carnoy solution or ethanol from the sample.
5. Thaw a centrifuge tube (freezer) containing 500 µl of sterile 0.3M mannitol pH 4.
6. Prepare the final DAPI stain by adding 25 µl of DAPI working solution (#3 see Appendix) to the 500 µl mannitol. Mix well.
7. Add 20 µl of this prepared final DAPI stain to the sample and mix well.
8. Store prepared samples for at least 5 minutes in the dark prior to fluorescent microscope analysis.
9. Pipette entire sample to a slide, cover with a cover glass and count the microspores.
10. Determine the fluorescence quality of the microspores, the number of mono- and bi- nucleate microspores, as well as the symmetrical bi-nucleate and multi-nucleate microspores (see attached sheet in appendix for details).

I. Appendix

1. Preparation of DAPI stock solution

- Prepare a 1 mg/ml stock solution by e.g. adding 5 mg of DAPI to 5 ml of sterile distilled water.
- Transfer aliquots to small tubes and keep dark by wrapping tube in foil.
- Keep stock frozen until needed.

2. Preparation of DAPI working stock solution

- To prepare the DAPI working stock solution, take 100 µL of the DAPI stock (see chapter 1 above) solution from above and add 900 µl of sterile distilled water.
- Keep 1 ml of working stock per centrifuge tube.
- Wrap tube in foil.
- Store in a freezer until needed.
- Keep one tube in a fridge for current use.

3. Preparation of final DAPI solution for DAPI analysis

- Transfer 500 µl of sterile 0.3 M mannitol pH 4 to centrifuge tube.
- Keep batches frozen until needed.
- Thaw one tube of 0.3 M mannitol and add 25 µL of DAPI working stock solution (see chapter 2 above).
- Mix well.
- Use prepared mixture on the same day. It can be kept in a fridge in darkness for up to 24 hours.

4. Buffered Hypochlorite Solution

- 20 ml, 10% w/v KH_2PO_4 Stock
- 20 ml Javex™
- Dilute to 1Litre with Milli-Q™ water
- 3 drops of Tween 80™
- pH to neutral

Note: This solution has a limited shelf life and should be made fresh when possible

5. Chickpea Microspore Induction Medium 1 (ChM1)

Macro in mg/L	ChM1 Medium	Stock solution	Stock #	Add	Done?
NH_4NO_3	1.000 mg				
KNO_3	1.900 mg				
$CaCl_2$ $2H_2O$	440 mg				
$MgSO_4$ $7H_2O$	370 mg				
KH_2PO_4	170 mg				
Micro in mg/L					
NaFeEDTA	40.00 mg	mg/ml		ml	
KI	0.83 mg	1 mg/ml		830 µL	
H_3BO_3	6.20 mg	1 mg/ml		6.2 ml	
$MnSO_4$ H_2O	16.91 mg	10 mg/ml		1.691 ml	
$ZnSO_4$ $7H_2O$	10.00 mg	10 mg/ml		1.0 ml	
Na_2MoO_4 $2H_2O$	0.25 mg	1 mg/ml		250 µL	
$CuSO_4$ $5H_2O$	0.05 mg	1 mg/ml		50 µL	
$CoCl_2$ $6H_2O$	0.025 mg	1 mg/ml		25 µl	
Vitamins					
Myo-Inositol	100.00 mg				
Pyridoxine HCl	0.50 mg	1 mg/ml		500 µL	
Thiamine HCl	0.50 mg	1 mg/ml		500 µL	
Nicotinic acid	5.00 mg	5 mg/ml		1000 µL	
Amino Acids					
Casein hydrolysate	200.00 mg				
Carbohydrates					
Sucrose g/L	90.00 g				
Plant Growth Reg.					
2,4-D mg/L	1.00 mg	1 mg/ml		1000 µL	
Picloram mg/L	0.25 mg	1 mg/ml		250 µL	
BAP	0.10 mg	1 mg/ml		100 µL	

PH	5.8	KOH			

Make up to 1 L.
Filter-sterilize.

Chickpea Experiment No.:

Donor Plant Information:

Genotype: _____ Batch No.: _____

Date of Planting: _____ Harvest No.: _____ Photoperiod: _____

Light Intensity: _____ Temperature: _____ Humidity: _____

Growth Chambers: _____

Condition of Plants: _____

Experiment Information:

Objective:

Pre-Treatments:

Type: _____ Location: _____

Length: _____ Condition of buds: _____

Extraction Information:

Extraction Medium: _____

Number of Buds: _____ Size of Buds: _____

No. of Microspores: _____ Microspore Density: _____

Medium Information:

Medium 1: _____ Liquid/Solid: _____

Medium 2: _____ Date of Transfer: _____

Incubator Information:

Incubator No.: _____ Temperature: _____

Photoperiod: _____

Comments:

Equipment List

- Ice bucket with ice
- Plastic container with lid (non-sterile)
- Tape and pen
- Ultra fine forceps (non-sterile)
- Deep dish Petri® dish with moistened filter paper (non-sterile)
- Paper towels
- Buffered hypochlorite solution with Tween
- Parafilm®
- Forceps with blunt tips (sterile)
- 1 L distilled water (sterile)
- Small Blender Cup
- ChM1 (sterile)
- Filter (sterile) for rinsing
- One 100 µm filter (sterile)
- One 35.5 µm filter (sterile)
- One 5 µm filter (sterile)
- Pasteur® and plastic pipettes (sterile)
- 100 µl and 5 ml pipettors with sterile plugged tips
- 15 and 50 ml sterile centrifuge tubes
- 500ml vacuum flasks (Stoppers included)
- Deep dish Petri® dishes (sterile)
- Haemocytometer
- Carnoy solution
- Sterile paper filter
- 2 ml centrifuge tube
- Slide with large cover slip
- 0.3 M mannitol pH 4
- DAPI working stock

DAPI Evaluation

Date:

Exp. #	Geno-Type	Day	Treatment	Fluores-cence	Mono-nucleate	Bi-nucleate Diff. Same	Multi-nucleate	Multi-cellular	Comments

I. Project Photos

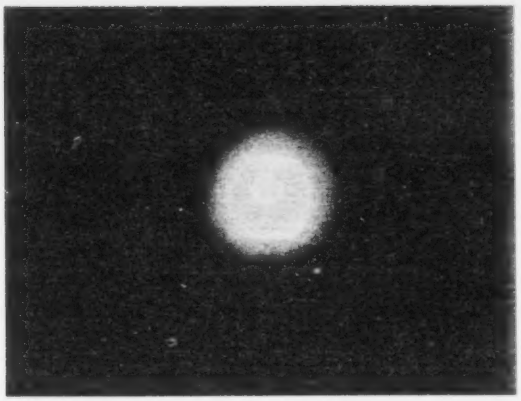


Fig. 3. Mono-nucleate haploid chickpea microspore.

Heat Pre-treatment prior to 1st Mitosis

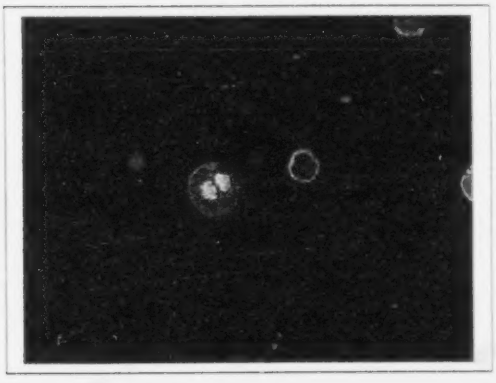


Fig. 4. Bi-nucleate microspore with symmetrical nuclei division

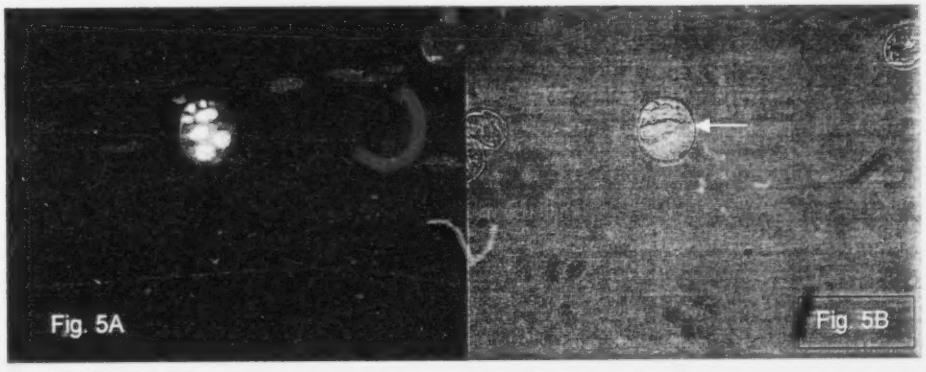


Fig. 5. Multi-nucleate microspore and beginning of microspore division. A. Fluorescent light only. B. Fluorescent and backlighting

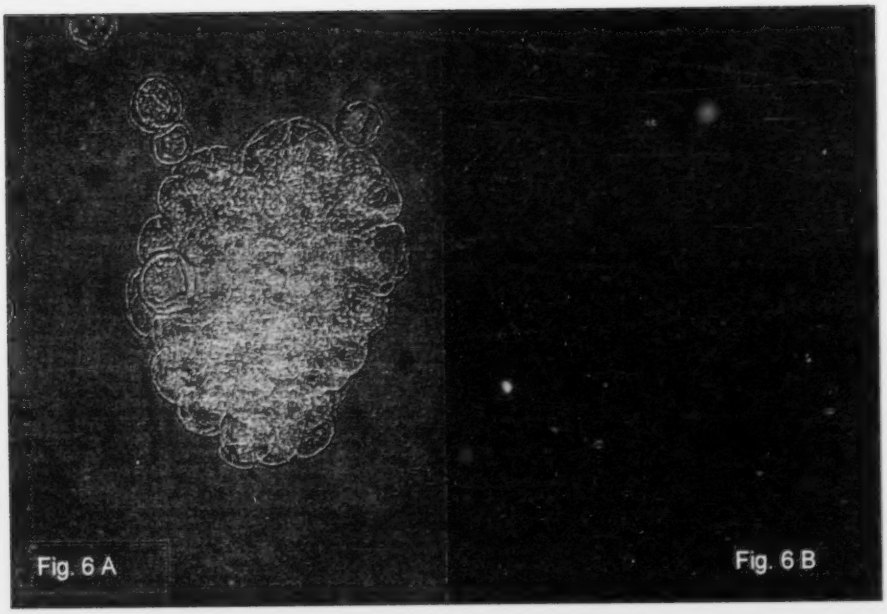


Fig. 6. Multi-cellular pro-embryogenic cluster. A. With fluorescent and backlighting. B. With fluorescent light only.

Fig. 7. Same multi-cellular clusters in liquid culture

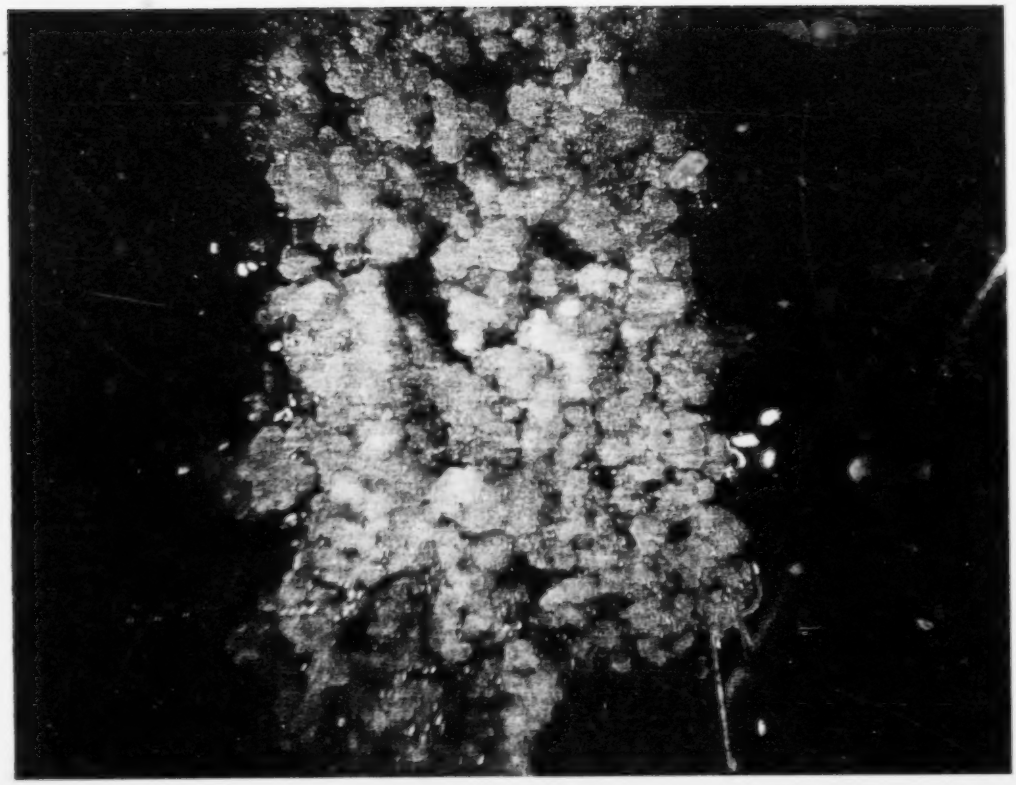


Fig. 8. Embryos derived from microspores callusing on plates